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Selection mosaics differentiate *Rhizobium*–host plant interactions across different nitrogen environments

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The nature and direction of coevolutionary interactions between species is expected to differentiate among distinct environments. Consequently, locally coevolved symbiotic traits would be well matched in similar environments, but mismatched elsewhere. In a classic mutualistic tradeoff, rhizobia provide nitrogen (N) to legume host plants in return for photosynthates. Despite earlier predictions, there is little evidence so far that spatial differences in soil N content mediate the coevolutionary outcome of the legume–*Rhizobium* mutualism. To test the existence of such selection mosaics, different genotypes of *Vicia cracca* and *Rhizobium leguminosarum* originating from spatially and environmentally highly differentiated sites were cross inoculated across different soil N regimes. In accordance with theoretical predictions, we found highly significant effects of genotype by genotype by environment (G × G × E) interactions, on both nodulation and plant growth, even when *R. leguminosarum* genotypes showed high genetic similarity. Our results show that the trajectory of the coevolutionary interactions between rhizobia and legumes is differentiated across different environments, and that selection mosaics may play an important role in shaping differences in the genetic composition of rhizobial populations.

The vast diversity of life is to a great extent a consequence of coevolution, since the competitiveness and survival of most organisms is imminently affected by their interaction with organisms from other species (Thompson 2005). In the last few decades, it has become increasingly clear that the nature and outcome of coevolutionary interactions between species may show high spatial variability (Thompson 1997, Forde et al. 2004, Decaestecker et al. 2007, Laine et al. 2014). These observations have resulted in the conceptual framework of the geographic mosaic theory of coevolution, of which the main hypothesis states that the outcome of reciprocal selection between a particular genotype of one species and a genotype of an interacting species, will differ among ecologically distinct locations (i.e. selection mosaics) (Thompson 1994, 2005). Consequently, locally coevolved symbiotic traits can be expected to be well matched only in similar environments, and mismatched in localities with different environmental conditions. Environmental change is therefore expected to destabilise the coevolutionary interactions within the affected communities (Six 2009, Warren and Bradford 2014).

A classic and well studied example of a coevolutionary interaction is the mutualism between Leguminosae and nitrogen (N) fixing bacteria known as rhizobia. Sheltered within root nodules, rhizobia fix atmospheric nitrogen (N_2) into ammonium (NH_4^+), providing the legume with a steady supply of N in return for photosynthates. However, when the costs of the mutualistic tradeoff exceed the benefits, selection will favour mutualists that will allocate less energy to their mutualistic partners (Neuhauser and Fargione 2004, Thompson 2005). For instance, when soil N is abundant, legumes will be less dependent on rhizobia and generally limit the formation of nodules (Streeter 1988, Naudin et al. 2011). In response, rhizobia evolve to become less efficient in fixing N (Weese et al. 2015). Hence, in agreement with the geographic mosaic theory of coevolution, geographical variation in soil N can be expected to result in geographical differences in how different genotypes of rhizobia and legumes interact. These differences can be tested using cross-inoculations, demonstrating a genotype by genotype by environment (G × G × E) interaction on response traits of both partners. Such G × G × E interactions are indicative of the existence of selection mosaics (Gandon and Nuismer 2009, Hoeksema 2010). Furthermore, G × G × E interactions could negatively affect the local fitness of immigrant rhizobia, and as such increase genetic differentiation among rhizobial populations (Parker 1999, Van Cauwenberghe et al. 2014).

Whereas selection mosaics has been demonstrated theoretically for the mutualism between rhizobia and legumes (Parker 1999), empirical evidence of a significant G × G × E interaction remains scarce, or is even absent to our knowledge. Barrett et al. (2012) tested for differences in the outcome of G × G × E interactions by combining *Acacia*

seeds and rhizobial communities from nine environmentally distinct locations for each of two *Acacia* species. The authors did not find significant interactions and suggested that the use of single strain inoculations would be more appropriate for testing differences in the outcome of G×G×E interactions. Heath et al. (2010) designed a cross inoculation experiment using different combinations of soil N-levels and *Sinorhizobium meliloti* and *Medicago truncatula* genotypes, which were sampled near French vineyards roughly 190 km apart (Heath 2010). They reported a marginally significant G×G×E interaction for nodulation, but found no such an effect for host plant traits.

The detection of G×G×E interactions requires the cross inoculation of mutualists originating from contrasting environments (Hoeksema 2010). This is especially the case when one of the partners, in this case *Rhizobium*, has strong dispersal capabilities and is limited in its local occurrence mainly by environmental selection (Baas-Becking 1934, Fierer and Jackson 2006). Such high gene flow can be expected to counteract geographic differentiation of the outcome of the interaction between rhizobia and legumes (Roberts and Cohan 1995, Nuismer et al. 1999). Furthermore, it is known that rhizobial populations are characterised by high genotypic diversity (Tian et al. 2010, Van Cauwenberghe et al. 2014), with genotypes differing in their efficiency to fix N (Burdon et al. 1999, Regus et al. 2015). These different genotypes belong to different clusters of conservative symbiotic plasmid and chromosomal housekeeping genes of which representatives have been found worldwide. Recently, it has been suggested that these genetic clusters within *R. leguminosarum* have little ecological relevance and that adaptive local genes, which can be exchanged through horizontal gene transfer (HGT), determine the ecological niche (Kumar et al. 2015). Alternatively, these genetic clusters could be clonal descendants from early diverged genetic lineages that represent ecological coherent groups (i.e. the ecotype model sensu Cohan 2002). The latter model implies that the use of rhizobia from different genetic clusters for testing differences in the outcome of G×G×E interactions might confound ecological differences among ecotypes with selection mosaics.

Here, we asked whether the mutualistic association between the leguminose *Vicia cracca* (tufted vetch or bird vetch) and its symbiont *Rhizobium leguminosarum* biovar *viciae*, is characterized by a geographic mosaic of coevolution. More specifically, we wanted to demonstrate a G×G×E interaction on a range of response traits of both partners, through exposing different *V. cracca* and *R. leguminosarum* biovar *viciae* genotypes from ecologically distinct regions to different soil N-levels. In accordance with theoretical predictions, we expect specific G×G combinations to form a more effective symbiosis under certain soil N-levels, while other combinations are suboptimal for one or both partners under these N-levels. *Vicia cracca* was chosen as our study system since it shows a relatively high level of strain specificity (Mutch and Young 2004), which may indicate strong coevolutionary interactions with its symbiont. To test our hypotheses, we set up an experiment with a full factorial design, combining three *V. cracca* genotypes and five *R. leguminosarum* biovar *viciae* genotypes, under two soil N conditions. The rhizobia and plants originated from populations of which we previously assessed the genetic composition of *R. leguminosarum* biovar *viciae* through sequencing two housekeeping genes and one symbiotic gene (Van Cauwenberghe et al. 2014, 2015). These populations were located in very distinct nutrient environments of variable degrees of agricultural intensification. Some of the rhizobial isolates used in this study belonged to the same genetic cluster of conservative symbiotic plasmid (*nodC*) and chromosomal housekeeping (*recA* and *glnII*) genes. We simultaneously tested whether G×G×E interactions still affect mutualist response traits when the inoculated rhizobial isolates belong to the same genetic cluster, but originate from different populations. Under the 'ecotype' model (Cohan 2002) one would expect isolates belonging to the same genetic cluster to represent identical ecotypes and show a similar trait response to different host plants and environments, whereas under the model of (Kumar et al. 2015), such genetic clusters of conservative genes would be of little ecological relevance. Finally, we also assessed the extent to which sympatric combinations of *Rhizobium* and *V. cracca* genotypes perform better than allopatric combinations.

Material and methods

Seed sampling

Vicia cracca seeds were sampled from three environmentally distinct populations of which the rhizobial genetic composition was screened earlier (Van Cauwenberghe et al. 2014, 2015): 'W' was located next to a cattle pasture in a landscape with high levels of soil eutrophication in West-Flanders (Belgium); 'B' was located in a road verge bordering a small forest in the more natural valley of the Dyle river in Brabant (Belgium); 'SV' was located in a species rich road verge in the mountainous and rather pristine southern Vosges (France). These populations were separated by large distances: min 96.09 km, max 425.34 km (Supplementary material Appendix 1 Table A1). Previously measured regional averages of soil macronutrients (Van Cauwenberghe et al. 2015), were high for population W, intermediate for population B, and low for population SV (Supplementary material Appendix 1 Table A2). Regional atmospheric N deposition levels were higher for population W than for populations B and SV (Supplementary material Appendix 1 Table A2). In each population, *V. cracca* seeds were harvest from ca 10 neighbouring individuals, in order to limit the within population genetic diversity of the sampled seeds, relative to the genetic diversity among the three sampled populations, which are unlikely to be connected through pollen or seed flow. Seeds from the three sampled populations are therefore further considered to represent three different *V. cracca* genotypes.

Selection of rhizobial isolates

From each of the three *V. cracca* populations, we selected a *Rhizobium leguminosarum* biovar *viciae* isolate representing the sequence type (ST) that occurred most frequently in this population. Over 580 isolates from West-Flanders, Brabant and the Vosges mountains were assigned to different

STs based on Sanger sequencing of the plasmid-borne gene *nodC* and the chromosomal genes *glnII* and *recA* (Van Cauwenberghe et al. 2015). The STs that were chosen for this study were W_ST81-C23, B_ST46-C28 and SV_ST74-C19, from populations W, B and SV, respectively (Supplementary material Appendix 1 Fig. A1–A2). Two additional isolates from the same ST, W_ST28-C11, from population W; and NV_ST28-C11, from a population in the northern Vosges (NV), were used to test whether rhizobia from different regions interact differently with different plant genotypes under various N-levels, despite high genetic similarity. The genetic distances among all isolates used in this study are displayed in the Supplementary material Appendix Fig. A1–A2, as a NeighborNet network (Bryant and Moulton 2004), built using SplitsTree ver. 4.12.8 (Huson and Bryant 2006).

Experimental setup

A full factorial experiment was set up, using *V. cracca* seeds from the three different populations, the five *R. leguminosarum* biovar *viciae* isolates, and two soil nitrogen (N) levels. For each plant genotype × soil N combination there was an uninoculated control treatment. Seeds were scarified to induce germination. Subsequently, seeds were surface sterilised by 1 min. submersion in 70% ethanol before 5 min submersion in 15% sodium hypochlorite. Finally, seeds were rinsed 6 times with sterile water and put on water agar plates for germination. *Rhizobium* genotypes were grown overnight in tryptone yeast extract (TY) medium at 30°C. Cell density was equalised by determining OD_{670}. Cell cultures were diluted in 10 mM $MgSO_4$ to obtain a cell density of about 10^8 cells ml^{-1}. Equal amounts of a N free nutrient solution (Broughton and Dilworth 1970) and water were added to all 400 ml pots which were filled with a 50:50 mixture of air dried river sand and perlite. Pots received different N-levels which were based on N-levels measured from natural populations (Van Cauwenberghe et al. 2015). N-levels were: low (< 5 mg NH_4NO_3 kg^{-1} soil) and high (50 mg NH_4NO_3 kg^{-1} soil). Initially, we imposed a third soil N condition, using 300 mg NH_4NO_3 kg^{-1} soil. However, this amount of N led to high plant stress and mortality, hence the results of this treatment were omitted from further analysis and discussion. Prior to inoculation, the pots were autoclaved and put in ethanol sterilised propagators. One seedling and 1 ml of diluted cell culture were added per pot. Within each propagator, pots were separated by sterilised bags and transparent cylinders. Plants were grown in a greenhouse using a day/night cycle of 16h/8h and a minimum light intensity of 200 W m^{-2}. No additional watering was needed and propagators were randomised weekly. Plants were harvested after eight weeks of growth. No nodule formation was detected on the uninoculated control plants, which confirmed the effectiveness of our sterilisation protocol and the absence of cross contamination.

Measurements and statistical analysis

We measured the plant shoot length and dry weight of the root and the shoot and counted the number of tendrils, branches and nodules. Carbon and nitrogen concentrations of the shoots were measured using a CHN analyser. Phosphorus concentration of the shoots was quantified after nitric acid destruction and subsequent ICP-OES analysis. Variables with a normal distribution were analysed using the GLM procedure in SAS (SAS Inst.) with *Rhizobium* genotype, plant genotype and N concentration, and their interactions as fixed factors. Similarly, the GENMOD procedure with log link function was used to analyse count variables following negative binomial distributions. Negative binomial distributions were tested against Poisson distributions and were shown to fit the data better. Pairwise differences between treatments were tested using a posteriori Tukey HSD test or the Bonferroni procedure, respectively. Applying mixed models using propagator as a random factor did not significantly alter our main results and are not further discussed (Supplementary material Appendix 1 Table A3). Data from uninoculated controls were excluded from the main analyses.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.v24v1> (Van Cauwenberghe et al. 2016).

Results

Nodulation response

The number of nodules found on each plant differed significantly among the different *Rhizobium* genotype × plant genotype × soil N combinations ($p < 0.0001$, F-value see Table 1, Fig. 1). Significant effects of soil N on nodulation were always negative, but the strength of this effect differed largely for different *Rhizobium* genotype × plant genotype combinations. The strongest decrease in nodulation was experienced by *Rhizobium* genotype B_ST46-C28 associated with SV plants (–92.5%, $p < 0.0001$). In contrast, W plants only suppressed nodulation with B_ST46-C28 with –22.8% ($p = 1.000$). Although NV_ST28-C11 and W_ST28-C11 show high genetic similarity, NV_ST28-C11 was less able to form nodules than W_ST28-C11 ($\bar{x} = 17 \pm 2$ and $\bar{x} = 26 \pm 3$, respectively, $p = 0.002$). However, their relative nodulation capacity depended largely on the N condition and the plant genotype with which they were associated ($\chi^2 = 22.86$, $p < 0.0001$). Also the nodulation capabilities of the sympatric rhizobia W_ST81-C23 and W_ST28-C11 depended on the interaction between N conditions and host plant genotype ($\chi^2 = 10.53$, $p = 0.005$). Combinations from *Rhizobium* genotypes and plant genotypes obtained from the same populations (sympatric combinations) formed on average significantly more nodules ($\chi^2 = 4.41$, $p = 0.038$, Supplementary material Appendix 1 Fig. A3) than allopatric combinations.

Plant response

Both tendril number and the number of shoots differed significantly among different *Rhizobium* genotype × plant genotype × N combinations ($p = 0.011$ and $p = 0.009$, respectively; Fig. 2 and Supplementary material Appendix 1 Fig. A4, respectively; Table 1). Plant dry weight, however,

Table 1. F-values from general linear models for normal distributed data and χ^2 values from generalised linear models with log link function for data which follow a negative binomial distribution, are shown for plant and mutualistic traits for five *R. leguminosarum* biovar *viciae* genotypes and three *V. cracca* genotypes grown under two contrasting N conditions lineages in all possible factorial combinations. DF = degrees of freedom. *: $0.05 \geq p > 0.01$; **: $0.01 \geq p > 0.001$; ***: $0.001 \geq p$.

	DF	Shoot length	Dry weight shoot	Dry weight root	Plant N	Plant C	Plant P	Tendril no.	Shoot no.	Nodule no.
N	1	1.04	8.75**	43.22***	4.23*	4.51*	3.99*	2.57	2.95	50.77***
Rhizobium genotype	4	2.68*	2.52*	4.77**	5.06***	2.64*	1.62	16.21**	30.23***	20.43***
Plant genotype	2	14.88***	12.31***	8.75***	0.66	0.28	0.48	0.33	6.12*	9.14*
Rhizobium genotype × N	4	1.41	2.04	4.42**	0.89	1.78	1.14	22.01***	11.92*	22.10***
Plant genotype × N	2	0.61	2.75	2.68	3.52*	2.41	0.60	1.58	5.65	30.64***
Rhizobium genotype × Plant genotype	8	0.51	2.28*	2.35*	0.86	0.89	1.05	11.39	17.24*	24.70**
Rhizobium genotype × Plant genotype × N	8	0.68	1.57	0.97	1.56	0.78	0.71	20.52**	19.91*	35.90***
Error DF		207	207	207	202	202	197	207	207	207

Figure 1. Mean (± SE) number of nodules formed by *V. cracca* genotypes from populations W, B and SV, either uninoculated (control) or in association with *R. leguminosarum* biovar *viciae* genotypes W_ST81-C23, B_ST46-C28, SV_ST74-C19, NV_ST28-C11 or W_ST28-C11 in soils with low (upper plot) and high (lower plot) N conditions.

remained unaffected by this three-way interaction (Table 1). Shoot and root dry weight did differ significantly among different *Rhizobium* genotype × plant genotype combinations ($p = 0.024$ and $p = 0.019$, respectively; Fig. 2 and Supplementary material Appendix 1 Fig. A5, respectively; Table 1). For instance, *V. cracca* plants from the SV population acquired a significantly higher shoot mass when associated with *Rhizobium* genotype SV_ST74-C19, than when associated with *Rhizobium* genotype B_ST46-C28 ($p = 0.002$). On the other hand, there was no significant difference in the effect of these genotypes on the shoot dry weight of *V. cracca* plants from population B or W ($p = 1.000$). The effect of these G × G interactions was independent of the soil N concentration which itself had an overall positive effect on shoot and root production ($p = 0.004$ and $p < 0.0001$, respectively; Table 1). Both *Rhizobium* genotype and plant genotype independently affected shoot length ($p = 0.033$ and $p < 0.0001$, respectively; Supplementary material Appendix 1 Fig. A6, Table 1). Also plant N and C content depended on the nodulating *Rhizobium* genotype ($p = 0.0007$ and $p = 0.035$, respectively; Supplementary material Appendix 1 Fig. A7–A8, respectively; Table 1). Plant P content was only affected by soil N ($p = 0.047$; Supplementary material Appendix 1 Fig. A9; Table 1) Soil N had different effects on

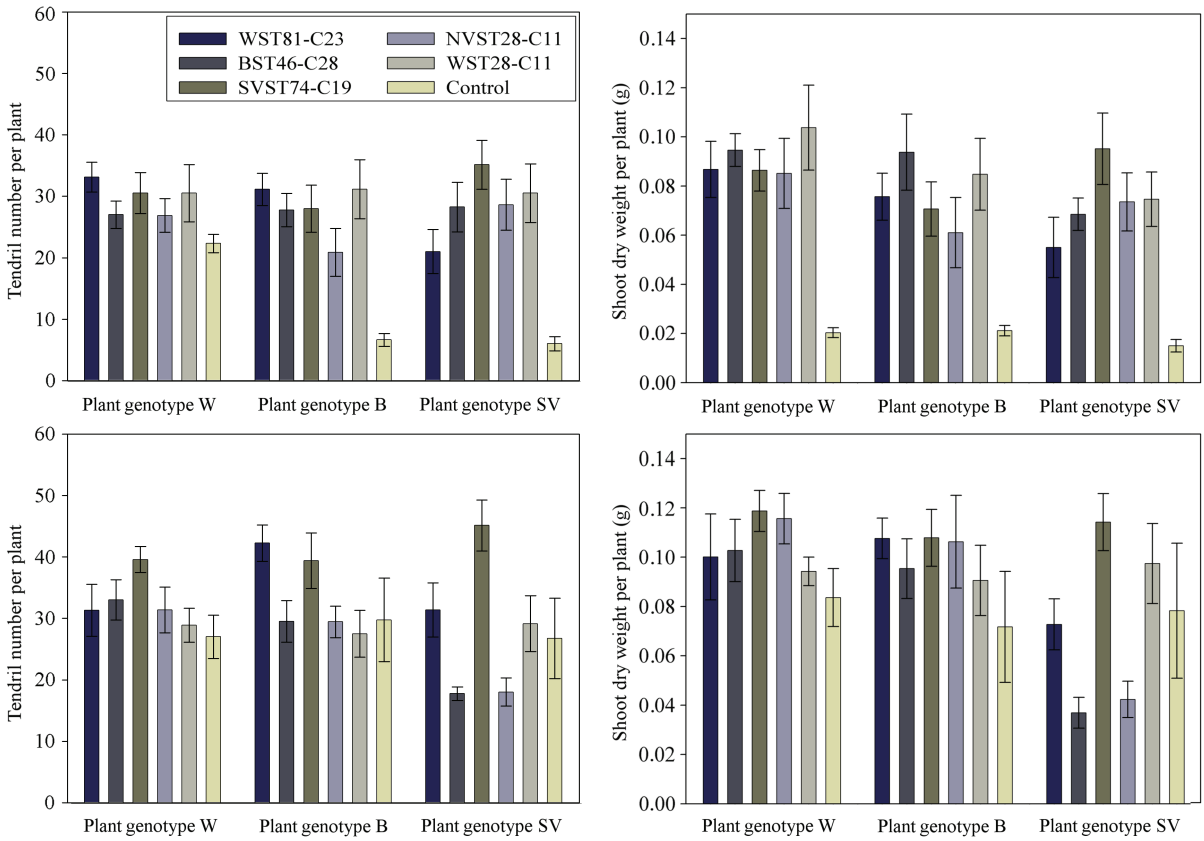

Figure 2. Mean (± SE) number of tendrils (left) and mean (± SE) shoot dry weight (g) (right) formed by *V. cracca* genotypes from populations W, B and SV, either uninoculated (control) or in association with *R. leguminosarum* biovar *viciae* genotypes W_ST81-C23, B_ST46-C28, SV_ST74-C19, NV_ST28-C11 or W_ST28-C11 in soils with low (upper plot) and high (lower plot) N conditions.

plant N for different plant genotypes ($p = 0.031$; Table 1). Although the allopatric W_ST28-C11 and NV_ST28-C11 *Rhizobium* genotypes belonged to the same ST, they differed in their effect on shoot number and shoot dry weight for different plant genotype × N combinations ($\chi^2 = 7.51$, $p = 0.023$ and $F_{2,92} = 4.17$, $p = 0.019$, respectively). In contrast, the sympatric but genetically distinct W_ST81-C23 and W_ST28-C11 *Rhizobium* genotypes did not show significant differences in their effect on any of the measured plant traits ($p > 0.500$). Sympatric combinations of *Rhizobium* genotypes and plant genotypes formed significantly more tendrils ($\chi^2 = 5.03$, $p = 0.025$, Supplementary material Appendix 1 Fig. A3) and acquired significantly higher shoot dry weight ($F_{1,24} = 7.56$, $p = 0.006$, Supplementary material Appendix 1 Fig. A3) than allopatric combinations.

Discussion

Genotype × genotype (G × G) interactions are well recognised to be a driving force behind reciprocal selection between rhizobial and legume genotypes (Heath and Tiffin 2007, Laguerre et al. 2007). Based on a cross inoculation experiment with host plants and *Rhizobium* genotypes from environmentally highly differentiated populations, we show for the first time a highly significant effect of G × G × E interactions on the response traits of both mutualistic partners. The fact that G × G interactions are affected by environmental conditions provides strong evidence for the existence of a selection mosaic sensu Thompson (2005, Hoeksema 2010). As we have putatively defined *Vicia cracca* genotypes as plants grown from seeds collected in a small area from the same population, our findings can be considered to be conservative, as it is likely that there still is some degree of genetic variability among *V. cracca* plants from the same populations.

More specifically, we found that soil N had a strong negative effect on the number of nodules for some G × G combinations, but had little effect on other combinations. Host plants typically avoid nodulation when N is sufficiently available (Streeter 1988, Gan et al. 2004, Naudin et al. 2011), reducing unnecessary energetic costs under these conditions (Layzell et al. 1981). However, soil N does not suppress nodulation for all G × G combinations, which indicates that different host plants differ in their tolerance to N addition for different *Rhizobium* combinations (Heath et al. 2010, Regus et al. 2015). Furthermore, we found a strong G × G × E interaction effect on vegetative growth (the number of tendrils and shoots) of *V. cracca*. In agreement with the meta-analysis of Friesen (2012), we found positive correlations between nodule number and vegetative plant growth (results not shown). This indicates that G × G × E interactions most likely affect vegetative growth indirectly through the positive effect of nodulation, or possibly through interactions with plant hormonal regulation (Glick

et al. 2007, Heckmann et al. 2011). On the other hand, we found little difference in plant shoot and root dry weight and N, C and P content among different G× G× E combinations. We speculate that plants analysed in this study may have been harvested too early to have accumulated significantly different amounts of nutritional reserves. G× G interactions had a clear effect on shoot and root dry weight, which was independent of the soil N level. Probably the host plant compensates for the lack of N provided by one source (e.g. soil N) by tapping into another N source (e.g. N fixed by rhizobia) depending on its N needs when associated with a specific *Rhizobium* genotype.

Several rhizobial isolates used in this study belonged to distinct genetic clusters of conservative symbiotic plasmid (*nodC*) and chromosomal housekeeping (*recA* and *glnII*) genes, which have been detected across large geographic distances (Van Cauwenberghe et al. 2014, 2015). These isolates could be different ecotypes of which the ecology is defined by the genetic cluster to which they belong, rather than by local coevolutionary interactions (Cohan 2002). However, we found G× G× E effects on response traits of both mutualists, even when rhizobial isolates belonged to the same genetic cluster, but originated from distant locations. Hence, despite having a similar genetic background regarding conservative symbiotic and housekeeping genes, these rhizobia were differentiated in their interaction with different host plants and environments. In comparison, two rhizobial isolates that were retrieved from population W showed little difference in their effect on host plant response traits, although they did differ in their nodulation success. These observations support the view of Kumar et al. (2015), stating that there is no observable relation between the phenotype of *Rhizobium* and the genetic cluster to which it belongs. It appears that no matter their genetic framework of conservative housekeeping and nodulation genes, rhizobia co-adapt with their local host plants and environment, possibly by incorporating local (co-)adaptive genes (Shapiro and Polz 2014, Kumar et al. 2015).

Indeed, sympatric combinations of rhizobia and *V. cracca* appear to be locally co-adapted, as they generally formed significantly more nodules and tendrils, and a greater amount of plant biomass than allopatric combinations. Although locally adapted rhizobia are known to inhibit the nodulation of more efficient rhizobia in agricultural fields (Roughley et al. 1976, Thies et al. 1991, Lindström et al. 2010), evidence for local co-adaptation within the legume-*Rhizobium* system is ambivalent (Parker 1995, Burdon et al. 1999, Heath 2010, Barrett et al. 2012). However, incidents of maladaptation could be expected where there is high gene flow of mutualists among communities in which reciprocal selection is shaped differently (Nuismer et al. 1999, Thompson 2005).

That different *V. cracca* genotypes provide different nodulation opportunities to different *R. leguminosarum* genotypes, depending on environmental conditions, is likely an important factor in explaining variability in rhizobial population structure. Previously, we have reported that a significant but small part of the variability in *R. leguminosarum* composition of natural *V. cracca* populations was explained by differences in soil acidity (Van Cauwenberghe et al. 2015). However, most variability remained unexplained or was related to unmeasured spatially correlated variables (Van Cauwenberghe et al. 2015). Furthermore, in contrast to rhizobial community studies (Zhang et al. 2011, Cao et al. 2014), we did not find an effect of soil N on the *R. leguminosarum* composition of *V. cracca* populations. Here we show that the nodulation capacity of rhizobia does not only depend on soil N, but also on the way different plant genotypes respond to different soil N-levels. Hence the expected direct effect of soil N on the genetic composition of *R. leguminosarum* nodulating *V. cracca* populations could have been masked due to the inconsistent way different *V. cracca* genotypes nodulate with different *R. leguminosarum* genotypes. These G× G× E interactions might have caused much of the unexplained and spatially correlated compositional variability in rhizobial populations. Indeed, the existence of selection mosaics for the mutualistic interaction between legumes and rhizobia was expected to hamper mutualist migration and thus to create genetically differentiated populations (Parker 1999).

Our study has clearly indicated that the manner in which a particular genotype of *V. cracca* affects response traits of a genotype of *R. leguminosarum* biovar *viciae*, and vice versa, depends on the environment, which strongly indicates the existence of a geographic selection mosaic for the legume–*Rhizobium* mutualism, corresponding with the geographic mosaic theory of coevolution (Thompson 1994, 2005, Gandon and Nuismer 2009). Furthermore, these G× G× E interactions could play an important role in determining the genetic composition of rhizobial populations. Our experiment included genotypes from populations that were separated by roughly 100–400 km. G× G× E interactions may also be important at smaller spatial scales. Selection mosaics have been described for *Pseudomonas syringae* and their phages from communities separated by at most 450 m (Koskella and Brockhurst 2014). *Rhizobium leguminosarum* populations are known to show genetic differentiation at similar spatial scales (Van Cauwenberghe et al. 2014). Further research should assess whether selection mosaics within the *Rhizobium*-legume mutualism are active at smaller spatial scales, despite possible counteracting gene flow (Nuismer et al. 1999, Gomulkiewicz et al. 2007).

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Supplementary material (available online as Appendix oik-02952 at <www.oikosjournal.org/appendix/oik-02952>). Appendix 1.

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